

What is claimed is:

1. (currently amended) ~~A method of preparing an hsiRNA mixture, comprising: reacting a preparation of double stranded RNA (dsRNA) with an effective amount of a mutant RNase III to produce the hsiRNA mixture.~~

A method, comprising:

reacting a preparation of large double-stranded RNA (dsRNA) with an effective amount of a mutant RNaseIII to produce a heterogeneous mixture of fragments in which at least 15% of the fragments have a size of 18-25 nucleotides, wherein the at least 15% of the fragments are not substantially degraded in the presence of the effective amount of the mutant RNaseIII for at least 1 hour, the heterogeneous mixture being suitable for silencing gene expression (hsiRNA).

2. (original) A method according to claim 1, wherein mutant RNase III is contained in a magnesium or manganese buffer.

3. (original) A method according to claim 2 wherein the mutant RNase III has a mutation in the position corresponding to E38 in *E.coli* RNase III.

4. (original) A method according to claim 2, wherein the mutation is E38A, E38T, E38W or E65A in *E.coli* RNase III.

5. (currently amended) ~~A method of forming an hsiRNA mixture, comprising:~~

~~combining a large dsRNA with a mutant RNase III for an effective time period so as to cleave the large dsRNA to form a heterogeneous mixture of fragments in which at least 15% of the fragments have a size of 18-25 nucleotides, the heterogeneous mixture (hsiRNA mixture) being suitable for silencing gene expression wherein~~

~~(i) at least 90% of the large dsRNA is cleaved as determined by gel electrophoresis and ethidium bromide staining; or~~

~~(ii) at least 30% of the cleaved dsRNA which forms the hsiRNA mixture has a fragment size of 18-30 nt.~~

A method, comprising:

forming a heterogeneous mixture of fragments by incubating a large double-stranded RNA (dsRNA) with a mutant RNaseIII for an effective time for cleaving, in the presence of magnesium ions or manganese ions, at least 90% of the large dsRNA as determined by gel electrophoresis and ethidium bromide staining wherein at least 30% of the cleaved dsRNA has a fragment size of 18-30nt.

6. (currently amended) A method according to claim 5, wherein the effective time ~~period~~ is about 1 minute to 20 hours.

7. (cancelled)

8. (currently amended) A method according to claim 5, wherein ~~steps (i) and (ii)~~ the effective time is about 1 minute to 5 hours ~~are achieved after 5 hours.~~

9. (currently amended) A method according to claim 5, wherein the effective time is about 1 minute to 10 hours ~~steps (i) and (ii) are achieved after 10 hours.~~

10. (original) A method according to claim 5, wherein the mutant RNase III is E38A or E65A.
11. (original) A method according to claims 1 and 5, wherein the large dsRNA has a length of at least 50 nt.
12. (withdrawn) A method of down-regulating gene expression of a target gene, comprising:
 - (a) preparing a heterogeneous siRNA mixture containing dsRNA fragments from a preparation of large dsRNA by means of a mutant RNase III;
 - (b) causing dsRNA fragments from the siRNA mixture to degrade mRNA transcribed from the target gene; and
 - (c) down-regulating gene expression of the target gene.
13. (withdrawn) A method according to claim 12, wherein the mutant RNase III is E38A or E65A.
14. (withdrawn) A method according to claim 12, wherein at least one of step (a) and (b) occurs *in vivo*.
15. (withdrawn) A method according to claim 12, wherein at least one of steps (a) and (b) occurs *in vitro*.
16. (withdrawn) A method according to claim 12, wherein the *in vivo* step occurs in a eukaryotic cell.

17. (withdrawn) A method according to claim 16, wherein the eukaryotic cell is present in a mammal such that reducing expression of the one or more target genes cause a phenotypic change.

18. (withdrawn) A method of claim 16, wherein the phenotypic change provides a treatment for a disease in the mammal.

19. (withdrawn) A method according to claim 16, wherein the phenotypic change is an enhancement of a desired characteristic in the mammal.

20. (withdrawn) A method according to claim 16, wherein the phenotypic change is diagnostic for a selected phenotype.

21. (withdrawn) A method according to claim 16, wherein the reduced expression of a gene is a tool for analyzing a biochemical pathway in which the gene product functions.

22. (withdrawn) A method according to claim 21, wherein the biochemical pathway may be further analyzed in combination with a diagnostic reagent.

23. (withdrawn) A method according to claim 22, wherein the diagnostic reagent is one or more antibodies.

24. (withdrawn) A method according to 16, wherein the eukaryotic cell is present in a non-human animal.

25. (withdrawn) A method according to claim 16, wherein the eukaryotic cell is a component of a transgenic animal is created from a fertilized oocyte containing the DNA sequence.

26. (withdrawn) A method according to claim 12, wherein step (a) further comprises combining a first hsiRNA mixture with one or more additional hsiRNA mixture for down-regulating gene expression.

27. (withdrawn) A method according to claim 12, further comprising:
selecting individual siRNA fragments from hsiRNA mixtures and introducing the individual siRNA fragments into a eukaryotic cell for down-regulating gene expression.

28. (withdrawn) An hsiRNA mixture wherein at least 30% of the preparation comprises fragments having a size in the range of 18-30 nt, the mixture containing more than 10 different sequence fragments, the mixture being capable of down-regulating targeted gene expression in a cell wherein the targeted gene is selected from the group consisting of Akt1, 2, 3, Erk1, 2, Msk 1, p38, IRS1, PKR, PTEN, CREB, ERa, ERb, DAX, p53, DNMT1, DnMT3B, DnMT3A, TRIP, Rb, MeCP2, Caspase3, La, Furin, EGFP, RFP, Ffluc and Renilla luciferase.

29. (withdrawn) A composition, comprising: an RNaseIII having one or more mutations wherein one mutation is located at a position corresponding to E38 in *E.coli* RNase III in which the glutamic acid (E) has been mutated to an alanine (A).

30. (withdrawn) A composition according to claim 29, further comprising a large dsRNA.